US-PAT-NO: 4094097

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TITLE: Method for preparing a pesticidally resistant rhizobium and agronomic composition thereof

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Nutritional Requirements of Rhizobia. In order to study the metabolism of these antifungal chemicals by the fungicide-tolerant strains, it was necessary to replace the complex, yeast extract-containing YEM medium with a simple mineral salts medium amended with only one or two growth factors. Consequently, a modified Vincent's, supra, mineral salts medium containing the following macro- and micro-nutrients per liter of distilled water was prepared: 10.0 g mannitol, 1.0 g K.sub.2 HPO.sub.4, 1.0 g KH.sub.2 PO.sub.4, 1.0 g KNO.sub.3, 0.2 g NaH.sub.2 PO.sub.4.2H.sub.2 O, 0.18 g MgSO.sub.4, 0.13 g CaSO.sub.4.2H.sub.2 O, 0.10 g Fe(NO.sub.3).sub.2.9H.sub.2 O, 0.2 mg NaMoO.sub.4.2H.sub.2 O, 0.2 mg ZnSO.sub.4.7H.sub.2 O, 0.2mg H.sub.3 BO.sub.3, 0.2 mg MnSO.sub.4.H.sub.2 O, 0.015 mg CuSO.sub.4, and 0.001 mg Co(NO.sub.3).sub.2.6H.sub.2 O. Portions (15.0 ml) of this mineral saltsmannitol medium were placed in 25 ml test tubes, and 7 of these tubes were amended with 1.3 mg/1 of each of the following seven vitamins: p-aminobenzoic acid, pyridoxine phosphate, inositol(meso), thiamine mononitrate, calcium pantothenate, riboflavin, and biotin. The growth factors were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Each tube was then inoculated

with 0.1 ml of a cell suspension derived from a 48-hour culture of the rhizobia
that had been washed three times with 0.1 M phosphate buffer (pH 7.0). The
cell density was 0.10 as measured by a spectrophotometer, model Spectronic 20,
at a wavelength of 540 nm. Controls consisting of inoculated and uninoculated
mineral salts-mannitol media were also prepared. Each treatment was in
duplicate. All the tubes were incubated on a rotary shaker at 30.degree. C.
for two days, with a shaker speed of 180 rpm. Growth of bacteria in each tube
was measured turbidimetrically at intervals of three hours using a
spectrophotometer (Spectronic 20), and measuring optical density at 540 nm. As
shown in Table 8, the adapted cowpea Rhizobium strain KO4SRPR grew well in a
mineral salts-mannitol medium amended with calcium pantothenate, whereas growth
was somewhat slower in the same medium treated with other growth factors or
with no vitamins. Thiram-tolerant R. meliloti 87TR grew readily in the mineral
salts-mannitol broth amended with inositol(meso) or pyridoxine phosphate, while
spergon-resistant R. phaseoli 203CR grew reasonably well when the medium was
treated with either calcium pantothenate or thiamine mononitrate. Growth
turbidity was observed visually in the case of R. meliloti 87TR and R. phaseoli
203CR.

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TITLE: Nitrogen fixation regulator genes

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Despite the ability of certain plants to induce nitrogenase activity in a
symbiotic relationship with some species of Rhizobium, the genetic analysis of
biological nitrogen fixation has previously been confined to free living
nitrogen fixing organisms, in particular Klebsiella pneumoniae. There are 17
linked nitrogen fixation (nif) genes arranged in at least 7 transcriptional
units in the nif cluster of Klebsiella (Kennedy, C., Cannon, F., Cannon, M.,
Dixon, R., Hill, S., Jensen, J., Kumar, S., McLean, P., Merrick, M., Robson, R.
and Postgate, J. (1981) In Current Perspectives in Nitrogen Fixation (A. H.
Gibson, W. E. Newton, eds.) Canberra: Australian Academy of Science, pp.
146-156; and Reidel, G. E., Ausubel, F. M. and F. M. Cannon (1979) Proc. Nat.
Acad. Sci. U.S.A. 76: 2866-2870). Three of these genes, nifH, nifD and nifK
encode the structural proteins of the nitrogenase enzyme complex (viz. the
Fe-protein subunit (dinitrogenase reductase) and the .alpha.- and
.beta.-subunits of the Mo-Fe protein (dinitrogenase) respectively.
Dinitrogenase is an .alpha..sub.2 .beta..sub.2 tetramer in which the two
non-identical .alpha. and .beta. subunits have similar molecular weights of
55,000 to 60,000. Dinitrogenase reductase is a dimer of two identical subunits
each having a molecular weight around 35,000. These genes are linked on the

same operon in K. pneumoniae and are transcribed from a promoter adjacent to the nifH gene. A similar situation (nifHDK) was found in two fast-growing Rhizobia, R. meliloti (Ruvkun, G. B., et al. (1982) Cell 29: 551-559) and R. leguminosarum (Schetgens, T. M. P. et al. (1984) Identification and analysis of the expression of Rhizobium leguminosarum PRE symbiotic genes, p. 699, In C. Veeger and W. E. Newton (eds.) Advances in nitrogen fixation research. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague). In the slow-growing R. japonicum, it has been found that nifDK forms one operon and that nifH is located elsewhere on the genome (Fuhrmann, M. and H. Hennecke (1982) Mol. Gen. Genet. 187: 419-425). A similar observation was made with another member of the slow-growing rhizobia, Rhizobium sp. Parasponia: a nifH region was found not to be linked to nifD (Scott, K. F., et al. (1983) DNA 2: 141-148). Yet a different arrangement was detected in the cyanobacterium Anabaena sp. 7120, in which nifHD is separated from nifK (Rice, D., et al. (1982) J. Biol. Chem. 257: 13157-13163). The remainder of symbiotic genes contain information required for bacterial attachment, root hair curling, initiation and development of nodules and establishment of symbiotic relationships. In addition, regulatory sequences such as promoters, operators, attenuators, and ribosome binding sites are found adjacent to the coding regions. These regulatory sequences control the expression of the structural genes, i.e., the coding sequences downstream in the 3'-direction of the DNA reading strand.